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Lung Cancer 34 (2001) 333–339

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Genetic polymorphism of *XRCC1* and lung cancer risk among African–Americans and Caucasians

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Received 12 March 2001; received in revised form 7 June 2001; accepted 11 June 2001

Abstract

Reduced DNA repair capacity may influence susceptibility to lung cancer. XRCC1 plays an important role in base excision repair and in rejoining DNA strand breaks. In the *XRCC1* gene, two common polymorphisms induce amino acid changes in codon 194 and codon 399 and correlate with levels of genotoxic damage. We examined the relation between these two polymorphisms and susceptibility to lung cancer among 334 incident cases and 704 population controls of African–American and Caucasian ethnicity in Los Angeles County, California. African–American and Caucasian subjects smoking 20+ cigarettes/day and carrying at least one copy of the codon 194 variant allele were at somewhat decreased risk of lung cancer (African–Americans OR = 0.2, 95% CI 0.1–0.9; Caucasians OR = 0.5, 95% CI 0.2–1.1). Similarly, for the codon 399 polymorphism, there was some evidence of a decreased risk for the homozygous variant genotype among heavier smokers (African–Americans OR = 0.3, 95% CI 0.0–2.9; Caucasians OR = 0.4, 95% CI 0.2–1.0). These results suggest that genetic variation in *XRCC1* might contribute to lung cancer and may interact with the amount smoked. Published by Elsevier Science Ireland Ltd.

Keywords: XRCC1; Polymorphism; Susceptibility; Lung cancer

1. Introduction

While smoking is the major risk factor for lung cancer, family studies suggest a role for genetic susceptibility [1]. Wei et al. have reported that individuals with reduced DNA repair capacity are at increased risk of developing lung cancer [2]. Reactive oxygen species present in cigarette smoke induce DNA base damage and single strand breaks that are repaired through the base excision repair (BER) pathway [3]. Polymorphisms in genes coding for DNA repair proteins may play a role in susceptibility to environmental lung carcinogens.

The *XRCC1* DNA repair gene plays a role in base excision repair and in rejoining DNA strand breaks [4,5]. Shen et al. found two common coding polymorphisms: a C→T substitution at posi-

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0169-5002/01/$ - see front matter Published by Elsevier Science Ireland Ltd.
PII: S0169-5002(01)00256-2

tion 26304 of exon 6 resulting in an amino acid change Arg194Trp, and a G→A substitution at position 28152 of exon 10 yielding an amino acid change Arg399Gln [6]. These polymorphisms code for nonconservative amino acid substitutions and occur in evolutionarily conserved regions, which suggests potential functional relevance [6–8]. In phenotypic studies by Lunn et al. and Abdel-Rahman et al. the codon 399 Gln allele was associated with higher levels of genotoxic damage and the codon 194 Trp allele appeared to be protective [9,10]. Duell et al. found that the 399 Gln/Gln genotype was related to increased sister chromatid exchange frequencies among smokers [11]. A third coding polymorphism in exon 9 (Arg280His) was also identified by Shen and colleagues [6]; however, this variant allele occurs at low frequency and was not associated with levels of genotoxic damage in the phenotypic study by Lunn et al. [10].

Two studies have examined the association between polymorphisms of *XRCC1* and lung cancer risk, and the results are conflicting [12,13]. To date, there are no published studies of *XRCC1* genotype in relation to lung cancer risk among African–Americans.

To investigate the possible association between polymorphisms in codon 194 and codon 399 of *XRCC1* and susceptibility to lung cancer, we analyzed DNA samples from incident cases of lung cancer and population controls of Caucasian and African–American ethnicity in Los Angeles County, California. The two *XRCC1* genotypes were determined using a multiplex PCR-RFLP method and information on smoking and other factors associated with lung cancer risk was obtained by questionnaire.

2. Materials and methods

A detailed description of the methods of subject enrollment and study population has been published previously [14,15]. In brief, we enrolled incident cases of lung cancer diagnosed at a network of hospitals in Los Angeles County between 1 September, 1990 and 6 January, 1994. At enrollment, cases had to be within 7 months of diagnosis to avoid possible bias due to the selection of cases who had survived for a long period. Controls under age 65 were sampled from the Department of Motor Vehicles driver's license lists and those over 65 from lists of Medicare beneficiaries. We frequency matched on age, sex, and ethnicity. Eligibility criteria for the study were as follows: resident in Los Angeles County, aged 40–84, able to complete a questionnaire in English, and no prior history of cancer other than non-melanoma skin cancer. Subjects provided a blood sample and completed a questionnaire on risk factors for lung cancer. The protocol was reviewed and approved by the Human Subjects Committee at the University of Southern California and written informed consent was obtained from all subjects.

Peripheral blood lymphocytes were isolated, and DNA was extracted as previously described [14]. *XRCC1* genotypes for codons 194 and 399 were detected using a multiplex PCR-RFLP technique as previously described by Lunn et al. [10]. For quality control, both PCR-RFLP assays were repeated on 5% of the samples and the replicates were 100% concordant.

Among the 356 eligible cases and 731 eligible controls enrolled, we obtained a DNA sample adequate to assign genotypes for *XRCC1* codons 194 and 399 on 334 cases (154 African–American and 180 Caucasian) and 710 controls (247 African–American and 463 Caucasian). Among subjects with genotype data, information on smoking was unavailable on six controls and these subjects were excluded from all analyses leaving 334 cases and 704 controls.

Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by unconditional logistic regression using SAS software, version 8 (SAS Institute; Cary, NC). Odds ratios were adjusted for the frequency matching factors (age and sex) and for smoking by including continuous terms for cigarettes smoked per day, years since quitting smoking, and duration of smoking in years. Although smoking adjustment had little effect on the OR, because lung cancer is strongly related to smoking in this [14] and other studies we present all results adjusted for age, sex, and smoking. We tested for interaction on the multiplicative scale by entering product terms into the logistic regression models.

3. Results

The age distributions of case patients and control subjects were quite similar (Table 1). There was a slight deficit of female controls. As expected, cases had markedly greater smoking history than controls. Current smoking among controls was similar to that observed among persons of comparable age and ethnicity in a contemporary national survey [16].

For codon 194, the frequency of the variant allele among controls was 0.082 (95% CI, 0.057–0.108) for African–Americans and 0.059 (95% CI, 0.043–0.074) for Caucasians (Table 2). For the codon 399 variant allele, the frequency was 0.181 (95% CI, 0.146–0.216) in African–Americans and 0.361 (95% CI, 0.330–0.393) in Caucasians. These allelic frequencies were similar to those observed by Lunn et al. [10]. These genotype frequencies were in Hardy–Weinberg equilibrium for both polymorphisms in both ethnic groups. Because of these differences in allele frequencies, we present results by race.

Among African–Americans, subjects with the Arg/Trp genotype for codon 194 were at statistically significantly decreased risk of lung cancer relative to subjects with the Arg/Arg genotype (OR = 0.4, 95% CI, 0.2–0.8) (Table 2). We observed no gene dosage effect, but we had too few homozygotes (Trp/Trp) to reliably estimate the association in this category (two cases and two controls). Among Caucasians overall, we observed no material association between codon 194 genotype and lung cancer risk. Subjects with the codon 399 Gln/Gln genotype were at a non-statistically significant decreased risk of lung cancer in both ethnic groups (African–Americans OR = 0.6, 95% CI 0.2–2.3; Caucasians OR = 0.6, 95% CI 0.3–1.3).

When we limited the analysis to smokers (defined as having ever smoked at least 100 cigarettes) results were not appreciably altered but the odds ratios were in the same direction for the Arg/Trp and Trp/Trp genotypes (Table 2); therefore, we combined these categories for subsequent stratification by amount smoked. We examined the association between *XRCC1* genotype and lung cancer risk by number of cigarettes smoked per day divided at 20, the median for all smokers in the study (Table 3). Although numbers become small upon stratification, among African–Americans, the Trp allele appeared to be slightly more strongly protective for lung cancer among the heavier smokers (OR = 0.2, 95% CI 0.1–0.9). Caucasian heavier smokers carrying a Trp allele were also at reduced risk of lung cancer (OR = 0.5, 95% CI 0.2–1.1). An increased risk was seen in the Caucasian lighter smokers, but with wide confidence intervals. The *P* value for difference in OR for the Trp allele by smoking was 0.02 among Caucasians.

Table 1
Study population characteristics for case patients and control subjects

Variable	African–Americans		Caucasians	
	Cases	Controls	Cases	Controls
N[a]	154	243	180	461
Age (years), mean (S.D.)	63.0 (8.8)	62.9 (7.9)	64.2 (10.1)	62.3 (8.6)
Female, *N* (%)	66 (42.9)	75 (30.9)	74 (41.1)	160 (34.7)
Smoking status, N (%)				
Never	8 (5.2)	76 (31.3)	7 (3.9)	165 (35.8)
Past	41 (26.6)	90 (37.0)	64 (35.6)	213 (46.2)
Current	105 (68.2)	77 (31.7)	109 (60.6)	83 (18.0)
Smokers only, mean (S.D.)				
Cigarettes/day	18.4 (15.8)	16.0 (12.3)	24.2 (15.0)	24.2 (18.1)
Years smoked	41.7 (11.2)	35.7 (14.5)	41.2 (12.4)	29.6 (14.1)

[a] *N*, number of subjects.

Table 2
Lung cancer risk in relation to *XRCC1* codon 194 and codon 399 polymorphisms

XRCC1 polymorphism	African–Americans			Caucasians		
	Cases	Controls	OR (95% CI)[a]	Cases	Controls	OR (95% CI)[a]
Codon 194						
All subjects						
Total	154	243		180	461	
Arg/Arg	142	205	1.0	158	407	1.0
Arg/Trp	10	36	0.4 (0.2–0.8)	22	54	1.0 (0.5–1.8)
Trp/Trp	2	2	2.3 (0.2–25)	0	0	–
Arg/Trp + Trp/Trp	12	38	0.4 (0.2–0.9)	22	54	1.0 (0.5–1.8)
Smokers only						
Total	146	167		173	296	
Arg/Arg	136	140	1.0	152	258	1.0
Arg/Trp	9	25	0.3 (0.1–0.8)	21	38	0.9 (0.5–1.8)
Trp/Trp	1	2	0.7 (0.1–11)	0	0	–
Arg/Trp + Trp/Trp	10	27	0.4 (0.2–0.8)	21	38	0.9 (0.5–1.8)
Codon 399						
All subjects						
Arg/Arg	105	164	1.0	87	186	1.0
Arg/Gln	46	70	1.1 (0.7–1.9)	76	217	0.8 (0.5–1.2)
Gln/Gln	3	9	0.6 (0.2–2.3)	17	58	0.6 (0.3–1.3)
Smokers only						
Arg/Arg	100	117	1.0	83	123	1.0
Arg/Gln	43	43	1.1 (0.7–1.9)	73	137	0.8 (0.5–1.3)
Gln/Gln	3	7	0.6 (0.1–2.5)	17	36	0.7 (0.4–1.4)

[a] Odds ratios (OR) and 95% confidence intervals (CI) are adjusted for age, sex and smoking (amount of cigarettes smoked/day, years since quitting smoking, and duration of smoking in years).

For the codon 399 polymorphism, the Gln/Gln genotype was associated with reduced risk of lung cancer in both African–American (OR = 0.3, 95% CI 0.0–2.9) and Caucasian (OR = 0.4, 95% CI 0.2–1.0) heavier smokers (Table 3). Among Caucasian lighter smokers, odds ratios for the Arg/Gln and Gln/Gln genotypes were above one, but were not statistically significant. P values of difference in ORs by smoking category were 0.08 for Arg/Gln and 0.03 for Gln/Gln among Caucasians suggestive of a gene–smoking interaction. Similar patterns were obtained for the codon 194 and 399 polymorphisms when the data were divided according to pack years at 35, the median for all smokers in the study (data not shown).

We examined the ORs for the codon 194 and codon 399 genotypes in relation to lung cancer risk by histology (adenocarcinoma, squamous plus small cell carcinoma, and all other cancers combined). For the codon 194 genotype, a protective effect of the Trp allele was seen for all combinations of ethnicity and histology except for other cancers in Caucasians. For the codon 399 polymorphism, the numbers of Gln/Gln do not permit further stratification but protective effects were seen for both adenocarcinoma and other cancers (data not shown).

4. Discussion

This is the first published study of the association between polymorphisms of *XRCC1* and lung cancer risk among African–Americans. Overall, the Trp allele of codon 194 was associated with significantly reduced risk of lung cancer among African–Americans. Although numbers become sparse upon stratification, among heavier smok-

ers, we found a protective effect in both African–Americans and Caucasians. Although results are not statistically significant our data suggest a reduced risk of lung cancer among heavier smokers with the Gln/Gln genotype for codon 399 in both ethnic groups. These results suggest that the ability of XRCC1 to repair DNA damage may be more important in heavier smokers who would be predicted to have more smoking-induced adducts and single strand breaks than lighter smokers.

The protective effect of the codon 194 Trp allele observed in our study is consistent with several studies of tobacco-related cancers and *XRCC1* genotype. Ratnasinghe et al. observed a decreased risk of lung cancer associated with the presence of at least one variant allele (OR = 0.7, 95% CI 0.4–1.2) among Chinese tin miners [13]. The frequency of the variant allele among Chinese controls was 0.348 [13]. In the only other study of lung cancer, the codon 194 polymorphism was not measured [12]. Subjects with a codon 194 Trp allele were also at decreased risk for squamous cell carcinoma of the head and neck (OR = 0.8, 95% CI 0.5–1.3) [17] and for bladder cancer (OR = 0.6, 95% CI 0.3–1.0) [18].

The direct effect of the codon 194 polymorphism on function of the XRCC1 protein is unknown, but data exist on biomarkers of DNA damage. Lunn and colleagues observed that individuals who carried at least one copy of the codon 194 variant Trp allele were less likely to have detectable levels of aflatoxin-B_1 DNA (AFB_1-DNA) adducts [10]. Abdel-Rahman et al. reported a lower level of sister chromatid exchange in cells with the Arg/Trp genotype compared to cells with the Arg/Arg genotype in response to treatment with the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) [9]. These two findings, while they were not statistically significant, support the observation of a decreased risk for the Trp allele in our study of lung cancer and the previous studies of lung and other smoking-related cancers [13,17,18].

Table 3
Association between XRCC1 codon 194 and codon 399 genotype and lung cancer risk in smokers stratified by cigarettes smoked per day[a]

	African–Americans			Caucasians		
	Cases	Controls	OR (95% CI)[b]	Cases	Controls	OR (95% CI)[b]
Codon 194						
<20 Cigarettes per day						
Arg/Arg	74	81	1.0	45	90	1.0
Arg/Trp+Trp/Trp	6	14	0.5 (0.2–1.3)	12	12	2.7 (0.9–7.7)
20+ Cigarettes per day						
Arg/Arg	62	59	1.0	107	168	1.0
Arg/Trp+Trp/Trp	4	13	0.2 (0.1–0.9)	9	26	0.5 (0.2–1.1)
Codon 399						
<20 Cigarettes per day						
Arg/Arg	53	64	1.0	25	52	1.0
Arg/Gln	25	28	1.1 (0.5–2.2)	24	40	1.4 (0.6–3.0)
Gln/Gln	2	3	1.1 (0.2–7.2)	8	10	2.0 (0.5–7.4)
20+ Cigarettes per day						
Arg/Arg	47	53	1.0	58	71	1.0
Arg/Gln	18	15	1.0 (0.4–2.4)	49	97	0.6 (0.4–1.1)
Gln/Gln	1	4	0.3 (0.0–2.9)	9	26	0.4 (0.2–1.0)

[a] Analysis is limited to case patients and control subjects who reported having ever smoked at least 100 cigarettes.
[b] Odds ratios (OR) and 95% confidence intervals (CI) are adjusted for age, sex, and smoking (cigarettes smoked per day, years since quitting smoking, and duration of smoking in years).

There are few data on lung cancer for codon 399. Divine et al. reported a significantly increased risk for the Gln/Gln genotype relative to the Arg/Arg genotype for adenocarcinoma of the lung among 129 non-Hispanic white cases (OR = 2.8, 95% CI 1.2–7.9) [12]. However, they observed no increased risk among Hispanics (OR = 0.8, 95% CI 0.2–2.8) [12]. The frequency of the variant allele among controls was 0.296 for non-Hispanic whites and 0.347 for Hispanics [12]. They did not present data for stratification by smoking. In contrast, Ratnasinghe observed no association for the Arg399Gln polymorphism and lung cancer risk among Chinese tin miners [13]. The frequency of the variant allele among Chinese controls was 0.245 [13]. Similar to our findings for codon 399, protective effects of the Gln allele have been found in two studies of other smoking-related cancers—head and neck, and bladder. Subjects with the Gln/Gln genotype have been reported to be at decreased risk of cancers of the head and neck (OR = 0.1, 95% CI 0.0–0.4) [19] and bladder (OR = 0.7, 95% CI 0.4–1.3) [18]. In contrast, Sturgis et al. observed an increased risk of head and neck cancer associated with the Gln/Gln genotype (OR = 1.59, 95% CI 0.97–2.61) [17].

In studies examining biomarkers of DNA damage associated with the codon 399 polymorphism, Lunn et al. found that levels of both AFB_1-DNA adducts and glycophorin A variants were higher among subjects with the Gln allele [10]. Duell and colleagues reported that the mean sister chromatid exchange (SCE) frequency was higher in three current smokers homozygous for the codon 399 Gln allele than in 14 never smokers homozygous for the Arg allele [11]. Higher levels of NNK-induced SCE were observed in cells with the Arg/Gln or Gln/Gln genotypes relative to cells with the Arg/Arg genotype [9]. These studies of adducts and SCE [9–11] provide only indirect evidence that the codon 399 amino acid change might be associated with decreased repair capacity. Direct studies of effects on gene function have not been done. If the Arg399Gln amino acid change results in deficient DNA repair as suggested by the phenotypic studies, DNA damage would accumulate in cells that carry such variants. While one might expect that variants leading to diminished XRCC1 function would provide an increased risk of cancer due to accumulated levels of genotoxic damage, it is plausible that cells with extensive genomic damage may be at a decreased risk for neoplastic transformation by virtue of an increased tendency to execute apoptotic pathways. Since many genes are involved in detoxification of carcinogens and reactive oxygen species and in repair of DNA damage, an alternative hypothesis is that another polymorphic gene might be in linkage disequilibrium with *XRCC1* codon 399. A direct assay of DNA repair function examining the effect of the codon 399 polymorphism would provide more insight into the potential relevance of this polymorphism.

In this study of African–Americans and Caucasians, heavier smokers carrying at least one Trp allele for codon 194 were at a decreased risk of lung cancer. For codon 399, we found some evidence that the Gln/Gln genotype may reduce lung cancer risk among African–American and Caucasian heavier smokers. These findings suggest a role for XRCC1 in lung cancer risk and a possible interaction with the amount smoked, the major risk factor for lung cancer.

Acknowledgements

The authors would like to acknowledge the efforts of Jan Lowery, Lena Masri and Catherine Carpenter for project management; Corinne Singer, Regina Olivas-Ho, Kisha Barnes and Steven Grossman for enrollment of subjects and Grace Chiu, PhD (Westat, Research Triangle Park, NC) for computer programming. The authors wish to thank Mariana Stern and Ruth Lunn for critical review of the manuscript and for helpful discussion. Data collection was funded by grants 1RT-140 and 3RT-0403 from the State of California Tobacco Related Disease Research Program. Laboratory and data analysis was supported by project ZO1-ES-49017 from Division of Intramural Research at the National Institute of Environmental Health Sciences. Case ascertainment was supported in part by the California Public Health Foundation, subcontract 050-F-8709, which is supported by the California De-

partment of Health Services as part of its statewide cancer-reporting program mandated by Health and Safety Code Sections 210 and 211.3. The ideas and opinions expressed herein are those of the authors and no endorsement by the State of California, Department of Health Services, or the California Public Health Foundation is intended or should be inferred. Case ascertainment was also supported in part by the Division of Cancer Prevention and Control, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, under the assigned contract number N01-CN-25403.

References

[1] Gauderman WJ, Morrison JL, Carpenter CL, Thomas DC. Analysis of gene–smoking interaction in lung cancer. Genet Epidemiol 1997;14:199–214.
[2] Wei Q, Cheng L, Hong WK, Spitz MR. Reduced DNA repair capacity in lung cancer patients. Cancer Res 1996;56:4103–7.
[3] Wilson DM III, Thompson LH. Life without DNA repair. Proc Natl Acad Sci USA 1997;94:12754–7.
[4] Rice PA. Holding damaged DNA together. Nat Struct Biol 1999;6:805–6.
[5] Thompson LH, West MG. XRCC1 keeps DNA from getting stranded. Mutat Res 2000;459:1–18.
[6] Shen MR, Jones IM, Mohrenweiser H. Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans. Cancer Res 1998;58:604–8.
[7] Shen MR, Zdzienicka MZ, Mohrenweiser H, Thompson LH, Thelen MP. Mutations in hamster single-strand break repair gene XRCC1 causing defective DNA repair. Nucleic Acids Res 1998;26:1032–7.
[8] Lamerdin JE, Montgomery MA, Stilwagen SA, et al. Genomic sequence comparison of the human and mouse XRCC1 DNA repair gene regions. Genomics 1995;25:547–54.
[9] Abdel-Rahman SZ, El-Zein RA. The 399Gln polymorphism in the DNA repair gene XRCC1 modulates the genotoxic response induced in human lymphocytes by the tobacco-specific nitrosamine NNK. Cancer Lett 2000;159:63–71.
[10] Lunn RM, Langlois RG, Hsieh LL, Thompson CL, Bell DA. XRCC1 polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. Cancer Res 1999;59:2557–61.
[11] Duell EJ, Wiencke JK, Cheng TJ, et al. Polymorphisms in the DNA repair genes XRCC1 and ERCC2 and biomarkers of DNA damage in human blood mononuclear cells. Carcinogenesis 2000;21:965–71.
[12] Divine KK, Gilliland FD, Crowell RE, et al. The XRCC1 399 glutamine allele is a risk factor for adenocarcinoma of the lung. Mutat Res 2001;461:273–8.
[13] Ratnasinghe D, Yao SX, Tangrea JA, et al. Polymorphisms of the DNA repair gene XRCC1 and lung cancer risk. Cancer Epidemiol Biomarkers Prev 2001;10:119–23.
[14] London SJ, Daly AK, Cooper J, Navidi WC, Carpenter CL, Idle JR. Polymorphism of glutathione *S*-transferase M1 and lung cancer risk among African–Americans and Caucasians in Los Angeles County, California. J Natl Cancer Inst 1995;87:1246–53.
[15] London SJ, Daly AK, Leathart JB, Navidi WC, Carpenter CC, Idle JR. Genetic polymorphism of CYP2D6 and lung cancer risk in African–Americans and Caucasians in Los Angeles County. Carcinogenesis 1997;18:1203–14.
[16] Giovino GA, Schooley MW, Zhu BP, et al. Surveillance for selected tobacco-use behaviors—United States, 1900–1994. Mor Mortal Wkly Rep CDC Surveill Summ 1994;43:1–43.
[17] Sturgis EM, Castillo EJ, Li L, et al. Polymorphisms of DNA repair gene XRCC1 in squamous cell carcinoma of the head and neck. Carcinogenesis 1999;20:2125–9.
[18] Stern MC, Umbach DM, van Gils CH, Lunn RM, Taylor JA. DNA repair gene XRCC1 polymorphisms, smoking, and bladder cancer risk. Cancer Epidemiol Biomarkers Prev 2001;10:125–31.
[19] Watson MA, Olshan AF, Weissler MC, Bell DA. XRCC1 polymorphisms (Arg194Trp and Arg399Gln) and risk for head and neck cancer. Proc Am Assoc for Cancer Res 2000;41:430–1.